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Pathogenicity and transmission of virulent Newcastle disease virus from the 2018–2019 California outbreak and related viruses in young and adult chickens

Kiril M. Dimitrov[a], Helena L. Ferreira[a,b], Mary J. Pantin-Jackwood[a], Tonya L. Taylor[a], Iryna V. Goraichuk[a,c], Beate M. Crossley[d], Mary Lea Killian[e], Nichole Hines Bergeson[e], Mia Kim Torchetti[e], Claudio L. Afonso[a], David L. Suarez[a,*]

[a] Southeast Poultry Research Laboratory, US National Poultry Research Center, Agricultural Research Service, USDA, 934 College Station Dr., Athens, GA 30605, USA
[b] University of Sao Paulo, ZMV, FZEA, Pirassununga, São Paulo 13635-900, Brazil
[c] National Scientific Center Institute of Experimental and Clinical Veterinary Medicine, 83 Pushkinskaya Street, Kharkiv 61023, Ukraine
[d] California Animal Health and Food Safety Laboratory system, Davis Branch, University of California, 620 West Health Sciences Drive, Davis, CA 95620, USA
[e] National Veterinary Services Laboratories, Veterinary Services, US Department of Agriculture, Ames, 1920 Dayton Ave, Ames, IA 50010, USA

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ABSTRACT

In May of 2018, virulent Newcastle disease virus was detected in sick, backyard, exhibition chickens in southern California. Since, the virus has affected 401 backyard and four commercial flocks, and one live bird market in California, and one backyard flock in Utah. The pathogenesis and transmission potential of this virus, along with two genetically related and widely studied viruses, chicken/California/2002 and chicken/Belize/2008, were evaluated in both 3-week- and 62-week-old chickens given a low, medium, or high challenge dose. All three viruses were highly virulent causing clinical signs, killing all the chickens in the medium and high dose groups, and efficiently transmitting to contacts. The three viruses also replicated in the reproductive tract of the adult hens. Virus shedding for all viruses was detected 24 hours after challenge, peaking with high titers at day 4 post challenge. Although not genetically identical, the studied isolates were shown to be phenotypically very similar, which allows the utilization of the available literature in the control of the current outbreak.

1. Introduction

Newcastle disease (ND) is defined by the World Organisation for Animal Health (OIE) as an *Avian avulavirus 1* (whose isolates are known as avian paramyxovirus 1 and Newcastle disease virus) infection caused by viruses that have an intracerebral pathogenicity index of 0.7 or higher (2.0 is maximum) or a fusion cleavage site with multiple basic amino acids and phenylalanine at position 117 (OIE, 2012). These viruses belong to the order *Mononegavirales,* family *Paramyxoviridae*, and genus *Avulavirus* (Amarasinghe et al., 2018) and are an extremely diverse group with a wide host range and varying virulence. The viruses in wild birds and non-commercial poultry are often referred to as avian paramyxoviruses 1 (APMV-1) to differentiate them from viruses causing virulent infection in poultry, commonly named virulent Newcastle disease viruses (NDV). Virulent NDV (vNDV) are among the most important pathogens for poultry worldwide (Dimitrov et al., 2016d; Miller and Koch, 2013). The disease they cause devastates poultry flocks causing up to 100% mortality in naïve birds and significant economic losses from outbreak eradication and trade restrictions (de Almeida et al., 2013; Miller and Koch, 2013). The identification of vNDV in poultry is notifiable to OIE. The clinical signs and pathology of ND in poultry encompass a wide spectrum of disease, ranging from mild respiratory disease to severe systemic infection with high mortality, and is characterized by high transmissibility and rapid spread (Miller and Koch, 2013). Newcastle disease viruses, as any other RNA virus, are constantly evolving (Chare et al., 2003). Based on the analysis of the complete coding sequence of the fusion gene (F-gene) (Diel et al., 2012a), NDV isolates are separated into two major classes, I and II, and are further classified into at least 19 genotypes with many sub-genotypes (Courtney et al., 2013; Czeglédi et al., 2006; de Almeida et al., 2013; Dimitrov et al., 2016d; Snoeck et al., 2013; Susta et al., 2014).

Newcastle disease was first identified in the U.S. in 1944 (Beaudette et al., 1948); however, evidence suggests that the disease had already been present in the country as early as 1938 (Beaudette and Hudson,

* Corresponding author.
E-mail address: david.suarez@ars.usda.gov (D.L. Suarez).

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1956). Since the first identification, viruses of different genotypes have been isolated in the country (Brown and Bevins, 2017; Dimitrov et al., 2016d). Low virulence viruses, from both class I and class II are commonly detected in wild waterbirds, many of which likely represent a natural reservoir of these viruses. Low virulence viruses are also commonly found in domestic poultry and waterfowl, and the first was isolated in the U.S. in the 1940s (Goldhaft, 1980; Ramey et al., 2017, 2013). NDV that are virulent for chickens and cause ND are not commonly isolated in the U.S., where ND is considered a foreign animal disease in poultry. However, there are two examples of virulent viruses that are endemic in wild birds in the U.S. The first group includes viruses of genotype VI (often referred to as pigeon paramyxoviruses 1) which are maintained in and frequently isolated from Columbiform populations (pigeons, doves) worldwide, and the other group includes sub-genotype Va viruses, which are maintained in some cormorant populations in North America (Diel et al., 2012b; He et al., 2018; Kim et al., 2008; Rue et al., 2010). Although occasionally, and also under experimental conditions, both of these virus lineages can infect chickens and can cause clinical disease (Alexander et al., 1985; Diel et al., 2012b; Heckert et al., 1996; Sabra et al., 2017; Susta et al., 2011). While there have been sporadic reports of the pigeon and cormorant viruses infecting poultry in the U.S., none of them have caused a disease outbreak in commercial poultry operations.

Shortly after the implementation of the live attenuated ND vaccines in the U.S. in the early 1950's (e.g LaSota and B1 strains) and the development of mass application methods, the prevalence of ND declined, and the disease caused by the neurotropic strains (i.e. vNDV of genotype II) was eradicated from the U.S. (Dimitrov et al., 2016a; Goldhaft, 1980; Hitchner, 1975; Miller et al., 2010). Indeed, in the last 50 years, there have been only two major ND outbreaks affecting commercial poultry in the U.S. The first one was caused by a virulent viscerotropic virus ancestral to genotype VI, introduced in the country by the importation of infected parrots in the early 1970's (Burridge et al., 1975; Hanson et al., 1973; Kinde et al., 2005). The virus was introduced in New York, Florida, Texas, New Mexico, and California, but only transmitted among commercial poultry in southern California (Hanson et al., 1973; Utterback and Schwartz, 1973). Between 1971 and 1973, the spread of the virus between chicken flocks was extensive, and of the 391 infected premises, 337 (86.2%) were raising poultry (Burridge et al., 1975). The epizootic was eradicated after three years at a cost of 56 million USD through the destruction of 11.9 million birds (Kinde et al., 2005) (total costs estimated at 318 million in 2018 USD). The next major outbreak occurred during 2002–2003, also in southern California (Nolen, 2003b). This outbreak was first detected in Los Angeles among privately owned game fowl during early October 2002, with spillover into commercial poultry during December 2002 (Nolen, 2003a, 2003b). The state and federal disease eradication costs were 160 million USD with an estimated 121 million USD in trade losses, and the depopulation of 3.21 million birds (Hietala et al., 2004) (combined costs estimated at $384 million in 2018 USD). The outbreak was caused by a virulent NDV of sub-genotype Vb that was closely related to poultry viruses from Honduras during 2000 and Mexico from 1996 to 2000 (Pedersen et al., 2004).

In May 2018, a swab sample was submitted to the California Animal Health and Food Safety Laboratory (CAHFS) branch in San Bernardino by a veterinary practitioner treating diseased backyard chickens. In addition, a concerned owner submitted a chicken carcass, where lesions and laboratory testing were found to be consistent with ND (Carvallo et al., 2018). The diagnosis was confirmed by the National Veterinary Service Laboratories (NVSL), Animal and Plant Health Inspection Services (APHIS) of the U.S. Department of Agriculture (USDA). This was the first report of vNDV in the U.S. in over 15 years. Thus far, 401 backyard flocks (primarily chicken, two turkey, two mixed species, and one pigeon flocks), 4 commercial laying hens farms, one live bird market, and 2 feeder stores have been confirmed positive for vNDV in five California counties – San Bernardino, Los Angeles, Riverside, Ventura, and Alameda (USDA/APHIS, 2019). A single case in backyard chickens was confirmed in Utah in January 2019. Vigorous control efforts have been implemented to eradicate the disease and protect from further spread in commercial poultry, including depopulation, quarantine, enhanced surveillance and epidemiologic trace backs, and significant efforts on education and outreach (CDFA, 2018). The human resources committed to disease control efforts have been significant; more than 800 state and federal personnel have been deployed to the field (some more than once), over 109000 premises have been visited, and over 4000 quarantines have been placed over the course of the outbreak (personal communication Heather Allen, USDA, APHIS Veterinary Services, National Preparedness and Incident Coordination).

The ongoing ND outbreak in California represents a significant threat to poultry operations in the U.S. As evidenced by previous outbreaks, further spread of the disease could have costly socioeconomic and commercial consequences and can cost hundreds of millions of dollars. Although the virus that caused the California 2002–2003 ND outbreak has been widely studied, data for the California 2018 virus is urgently needed. The aim of this study was to investigate the pathogenesis, transmission, and shedding dynamics of the 2018 virus causing current outbreaks in southeastern California in both three- and 62-week-old chickens, along with related NDV that caused outbreaks in California in 2002–2003 and Belize in 2008. This was achieved through the evaluation and description of the: i) susceptibility of young and adult chickens to infection with a low, medium and high dose of the 2018 California vNDV; ii) side-by-side comparison to the related 2002 California and 2008 Belize vNDV; iii) pathogenesis and shedding dynamics of the studied viruses and their transmissibility to naïve contact chickens; and iv) phylogenetic relationships with other NDV. The obtained data demonstrate close genetic and phenotypic relationships between the three studied viruses and suggest that the existing literature for the characterization of the California/2002 and Belize/2008 viruses can be utilized to facilitate risk assessment and informed control efforts for the eradication of the disease in California.

2. Materials and methods

2.1. Viruses

Three NDV were used in this study. Two of the viruses (chicken/USA/CA/212676/2002 and chicken/Belize/4224–3/2008, CA02 and BE08 used hereafter, respectively) were obtained from the Southeast Poultry Research Laboratory's (SEPRL) repository of the United States National Poultry Research Center (USNPRC), USDA. The virus from the 2018 California outbreak (chicken/California/D1806566/2018, CA18 used hereafter) was kindly provided with CAHFS permission by NVSL, APHIS, USDA. All viruses were propagated in 9-to-11-day-old specific pathogen free (SPF) embryonating chicken eggs (ECEs) following standard procedures (Senne, 2008). Allantoic fluids from all viruses were diluted in brain heart infusion (BHI) medium (BD Bioscience, Sparks, MD) in order to prepare an inoculum with 10^2, 10^4, or 10^6 50% egg infective doses (EID_{50}) per 0.1 ml (low, medium, and high dose, respectively). All challenge doses were subjected to back-titration in ECEs, and the results are presented in Supplemental Table S1. The intracerebral pathogenicity index (ICPI) assay was conducted on CA18 per OIE recommendations (OIE, 2012). The ICPIs for the CA02 and BE08 viruses were previously reported as 1.79 and 1.75, respectively (Susta et al., 2014; Wakamatsu et al., 2006a).

2.2. Birds

Three-week-old and 62-week-old SPF birds were obtained from the SEPRL White leghorn in-house chicken flocks (*Gallus gallus domesticus*) (egg layer type). All birds were housed in self-contained isolation units that were ventilated under negative pressure with HEPA-filtered air. Birds were placed in the isolators and allowed to acclimate for at least

24 h prior to inoculation. Feed and water were provided with ad libitum access. All animal experiments were approved and performed under the regulations of the USNPRC's Institutional Animal Care and Use Committee in animal biosecurity level 3 enhanced (ABSL-3E) facilities at the SEPRL.

2.3. Experimental design

Two similar experiments were conducted, one with 3-week-old chickens (n = 83) and one with 62-week-old hens (n = 72). In each experiment, birds were separated into a negative tissue control group (n = 2), and nine infectivity/transmission groups (n = 5 per group, 3 groups per virus), each receiving a low, medium, or high dose of the CA02, BE08, or CA18 (see Supplemental Fig. S1). The viruses in the infectivity groups were administered using gavage needles, and the inoculum was delivered via the choanal cleft and the left eye of each bird. An additional group for examining the pathogenesis of CA18 was also included in each of the two experiments. The birds in these groups (n = 9 for young birds and n = 7 for adult birds) received a high dose of the CA18 virus using the same application method. At one, two, and three days post-infection (DPI), two to three birds from the pathogenesis groups were euthanized, necropsied, and gross lesions were recorded. The following tissues were collected in 10% neutral buffered formalin (NBF) for evaluation of microscopic lesions and virus replication: nasal turbinate, trachea, larynx, lung, air sacs, comb, eye lid, heart, brain, esophagus, proventriculus, duodenum, cecal tonsils, pancreas, liver, kidney, adrenal gland, spleen, Harderian gland, skeletal muscle, feathers, and testis. In addition, cloacal bursa and thymus were collected from the young chickens, and ovary, infundibulum, magnum, isthmus, and shell gland were collected from the reproductive tract of the adult hens. To determine the transmission potential of the viruses by exposure contact, at 2 DPI, three young or two adult naïve uninfected birds were placed in each of the infectivity/transmission groups (only two birds were placed in the adult groups due to IACUC guidelines for bird density). The birds were observed twice daily, and birds that showed severe clinical signs, stopped eating or drinking, or remained recumbent were euthanized and reported as dead on the next day for the calculation of mean death times. All euthanized birds were necropsied and gross lesions were recorded. The tissue samples described above were also collected from two birds from the infectivity/transmission groups euthanized due to severe clinical signs at either 4 or 5 DPI. Additionally, spleen, lung, brain, and reproductive tract tissues (adult birds only) were collected in sterile tubes from all birds necropsied at 4–5 DPI, and stored at −70 °C until further processing. Oropharyngeal (OP) and cloacal (CL) swabs were collected at 1, 2, 3, 4, and 7 DPI from directly inoculated birds, and at 2 and 5 days post-placement (DPP) from contact exposed birds to determine virus shedding. All surviving birds were bled at 16 DPI (14 DPP) for serology and were euthanized.

2.4. RNA extraction and quantitative rRT-PCR

RNA was extracted from swab medium using the MagMAX 96 AI/ND viral RNA isolation kit (Ambion, USA) with a KingFisher magnetic particle processor (ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Quantitative real-time RT-PCR (rRT-PCR) for NDV detection targeting the fusion (F) gene was performed using the AgPath-ID One-Step RT-PCR Kit (Ambion) and the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, USA) utilizing primers and probe described previously by Creelan et al. (F+4829) and Wise et al. (F-4939 and F+4894) (Creelan et al., 2002; Wise et al., 2004). The assay conditions were reverse transcription for 10 min at 45 °C; initial denaturation for 10 min at 95 °C; 40 cycles of denaturation for 10 s at 95 °C, primer annealing for 30 s at 58 °C, and primer elongation for 10 s at 72 °C. The total reaction volume of 25 µl for each sample consisted of molecular grade water – 2.15 µl; 2X buffer – 12.5 µl; forward primer – 0.65 µl (20 pmol/µl); reverse primer – 0.35 µl (20 pmol/µl); probe – 0.35 µl (6 pmol/µl); 25X enzyme mix – 1.0 µl; and RNA template – 8.0 µl. For virus quantification, a standard curve for each virus was established with RNA extracted and diluted from the same titrated stock of the viruses used to challenge the birds, and the results are reported as EID_{50}/ml. The calculated lower detection limit of the assay varied between $10^{1.5}$ and $10^{1.7}$ EID_{50}/ml.

2.5. Virus replication in tissues

Virus replication in spleen, lung, brain, and reproductive tract tissues (adult birds only), from 2 birds from each virus group infected with a high dose, were examined at 4 DPI, with the exception of one adult bird from the BE08 group from which tissues were collected at 5 DPI and one young bird from the each of the CA18 and CA02 groups which were inoculated with a medium dose. After thawing, the tissues were weighed, homogenized, and diluted in BHI to a 10% (weight/volume) concentration. Viral RNA was extracted using TRIzol LS reagent following manufacturer's instructions (Invitrogen, USA). Virus quantification was performed using quantitative rRT-PCR as described above.

2.6. HI assay

Hemagglutination inhibition (HI) assay was used to evaluate the presence of antibodies in serum samples collected from all surviving birds at the end of the experiments. Following standard procedures (OIE, 2012), the respective challenge virus was used as an antigen for each group of sera. Titers were calculated as the reciprocal of the last HI-positive serum dilution, and samples with HI titers of 8 and below were considered negative.

2.7. Microscopic lesions and viral antigen staining in tissues

Collected tissues were prepared for histopathology and immunohistochemistry (IHC) as previously described (Pantin-Jackwood and Swayne, 2007). Briefly, the tissues fixed by submersion in 10% NBF were routinely processed and embedded in paraffin. Sections were made at 4 µm and were stained with hematoxylin and eosin. Duplicate sections were immunohistochemically stained by first microwaving in CitriSolv Solvent (ThermoFisher Scientific, USA) for antigen exposure. A 1:8000 dilution of a primary polyclonal rabbit-derived anti-NDV nucleoprotein (raised against the synthetic peptide TAYETADESETRRIC) (Kommers et al., 2001) was applied and allowed to incubate overnight at 4 °C. The primary antibody was then detected by the application of biotinylated anti-immunoglobulin and using a phosphatase-conjugated biotin-streptavidin detection system (Super Sensitive Multilink IHC Detection System; BioGenex, USA). Fast Red TR (BioGenex) served as the substrate chromogen, and hematoxylin was used as a counterstain. All tissues were systematically screened for microscopic lesions and virus antigen staining. The intensity of viral antigen staining in each section was scored as follows: – = no IHC signal present; + = rare cells positive on IHC; + + = positive cells seen, < 50% of HPF (high-power field, ×400 magnification); + + + = positive signal seen in 50–75% of HPF; + + + + = abundant positive signal in > 75% of HPF.

2.8. Sequencing and phylogenetic analysis

RNA extraction, complete genome deep sequencing, and genome assembly for CA02 and BE08 viruses were performed as described previously (Dimitrov et al., 2017). RNA from the CA18 virus was extracted using MagMAX 96 Viral RNA Isolation Kit (Ambion) following manufacturer's instructions; cDNA was generated using sequence-independent single primer amplification (SISPA) (Chrzastek et al., 2017). Libraries were prepared using the Ion Xpress Plus gDNA Fragment Library Preparation Kit (ThermoFisher Scientific), and sequencing was performed using the Ion Chef and S5 next-generation sequencing system (ThermoFisher Scientific). The complete fusion gene coding sequences and the complete genome sequences of all available class II NDV isolates were downloaded from GenBank of the National Center for Biotechnology Information (Benson et al., 2017) as of September 18th, 2018, resulting in two preliminary datasets of 2352 and 471 sequences, respectively. The sequences in each dataset were aligned using Multiple Alignment with Fast Fourier Transformation (MAFFT v.7.221.3) (Katoh and Standley, 2013) as implemented in the Galaxy platform (Goecks et al., 2010). For the complete genomes, the leader and tail sequences and intergenic regions were trimmed, and the coding sequences of all six genes were concatenated together. The datasets were used to estimate the pairwise and the mean interpopulational distances (nucleotide distances between genetic groups). The estimates of average evolutionary distances were inferred using MEGA6 (Tamura et al., 2004). Analyses were conducted using the Maximum Composite Likelihood model. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). Next, a preliminary analysis was performed to infer the evolutionary history in each dataset (data not shown). Smaller groups (n = 62 and n = 68, respectively, Supplemental Tables S2 and S3) including the most closely related viruses and representative viruses from other genotypes were parsed from the initial datasets and further analyzed. Maximum-likelihood trees based on general time-reversible (GTR) model were constructed by using RaxML version 8.2.11 (Stamatakis, 2014) with 1000 bootstrap replicates. A discrete gamma distribution was used to model evolutionary rate differences among sites. Trees were visualized using FigTree (v.1.4.2) (Rambaut and Drummond, 2012). The Roman numerals presented in the taxa names in the phylogenetic trees represent the respective genotype for each isolate, followed by the GenBank accession number, host name, country of isolation, strain designation and year of isolation (if available). Previously described criteria (Diel et al., 2012a) based on the phylogenetic topology and evolutionary distances between different taxonomic groups were used to classify the studied isolates.

2.9. Accession numbers

The complete genome sequences (n = 3) of the three vNDV (CA02, BE08, and CA18) obtained in this study were submitted to GenBank and are available under the accession numbers MK040373, MK214317, and MK214318.

2.10. Statistical analysis

Data were analyzed using Prism (v.7.03) software and outliers were identified using the ROUT test (GraphPad Software Inc., USA). The D'Agostino-Pearson normality test was performed to estimate if the values in each group come from a Gaussian distribution. Based on the normality distribution, non-parametric Kruskal-Wallis test with Dunn's posttest were used for multiple comparisons of mortality rates and viral titers in oropharyngeal and cloacal swab samples from the same doses between different viruses within young and adult birds, and also the same viruses and doses between ages too. For statistical purposes, all swab samples from which viruses were not detected were given a numeric value of 1 log below the limit of detection for each virus. Statistical significance was set at a P value of < 0.05.

3. Results

3.1. Sequencing and phylogenetic analysis

To determine the genetic characteristics of the viruses used in this study isolated from California in 2002 and 2018 and Belize in 2008, we performed sequencing and evolutionary distance analysis of these isolates. The consensus sequences of all three isolates predicted fusion protein cleavage sites that contained three basic amino acids at positions 113–116 and a phenylalanine residue at position 117 ($_{113}$RQKR↓F$_{117}$). Based on criteria utilized by OIE to assess virulence of NDV isolates, such a cleavage site motif is specific for virulent viruses (OIE, 2012). The full fusion gene coding sequences of the studied isolates were further analyzed in order to determine the evolutionary distances among them and between them and other viruses. The virus causing the 2018–2019 California outbreak is closest genetically to a class II sub-genotype Vb NDV isolated from a chicken in Honduras in 2007 (98.2% nucleotide identity), BE08 and CA02 (97.9% and 97.4% nucleotide identity, respectively). The mean genetic distance of the CA18 virus when compared to seven additional sub-genotype Vb viruses isolated in the U.S. (Amazon parrots) and Mexico (chickens) in the last 20 years was 4.2% (ranged from 3.2% to 5.9%). Another six viruses from chickens from Honduras (2000) and Nicaragua (2001), and from parrots from the U.S. (1980s) were even less similar to the CA18 virus with mean nucleotide distances of 7.8% (that ranged from 6.0% to 9.1%). The mean evolutionary distance between the sub-genotype Vb and sub-genotypes Vc, Va, and Vd was 6.5%, 10.2%, and 11.2% respectively.

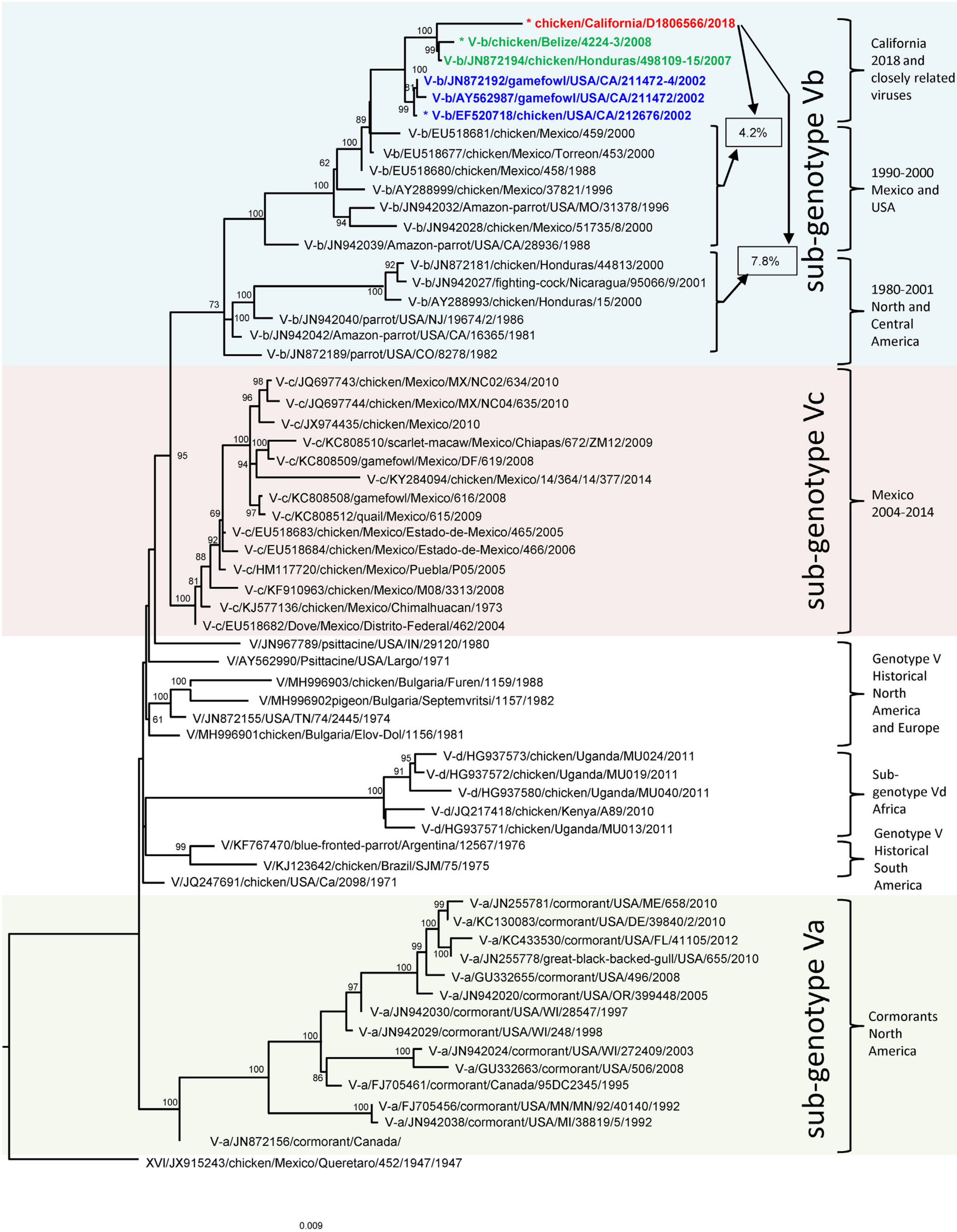
In order to determine the phylogenetic relationship between the studied viruses and other class II NDV available in GenBank, the complete fusion gene coding sequences obtained in the current work, along with sequences of closely related viruses, were used to construct a phylogenetic tree (n = 62) (Fig. 1 and Supplemental Table S2). A second phylogenetic analysis was performed using 68 complete genome concatenated coding sequences (see Supplemental Fig. S3 and Supplemental Table S3). Both, the full fusion gene and the complete genome phylogenetic analyses displayed similar topology, and following the classification and nomenclature criteria put forth by Diel et al. (Diel et al., 2012a), confirmed the phylogenetic classification of the viruses studied here into sub-genotype Vb of class II. The topology of the full fusion gene phylogenetic tree (Fig. 1) indicated that CA02, BE08, and CA18 grouped together with the viruses from the chickens and parrots from Honduras, U.S, and Mexico isolated between 1996 and 2007. While still within the sub-genotype Vb branch, the chicken viruses from Honduras (2000) and Nicaragua (2001), and from parrots from the U.S. (1980s) that showed higher genetic distance to the viruses studied here, clustered together in a separate monophyletic branch of the dendrogram (Fig. 1).

3.2. ICPI

The CA18 virus presented an ICPI value of 1.76. All three studied viruses had high ICPI values (CA02 and BE08 had 1.79 and 1.75, respectively), which characterizes them as virulent NDV based on the OIE standards (OIE, 2012). These values are concordant with the cleavage site motifs of the viruses; ICPI values above 0.7 are defined as virulent and above 1.5 are indicative of velogenic NDV (Alexander and Swayne, 1998).

3.3. Clinical signs in three-week-old chickens

The mortality induced by direct oculo-nasal infection with the viruses at three different challenge doses in 3-week-old chickens is depicted as survival curves in Fig. 2. No clinical signs or mortality were observed in the low dose groups inoculated with CA18 and BE08. Chickens inoculated with the high dose of CA02 (2 out of 5) and CA18 (7 out of 12) showed mild lethargy as early as 2 DPI. One chicken in the CA18 medium dose group and two chickens from the CA18 high dose group showed mild to moderate bilateral conjunctivitis on the same day. Between 3 and 5 DPI, all birds in the medium and high dose groups displayed clinical signs. The initial mild lethargy progressed to moderate and severe lethargy with the birds in the high dose groups being affected slightly earlier (Fig. 3A and B). All birds in these groups also developed bilateral conjunctivitis, ranging from mild to moderate in the medium dose groups, and from mild to severe in the high dose groups.



(caption on next page)

Fig. 1. Phylogenetic analysis based on the full-length nucleotide sequence of the fusion gene of isolates representing Newcastle disease virus class II, genotype V. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model with 1000 bootstrap replicates. The tree with the highest log likelihood (-9133.6828) is shown. A discrete gamma distribution was used to model evolutionary rate differences among sites. The analysis involved 62 nucleotide sequences with a total of 1653 positions in the final dataset. The viruses used in the animal experiments in this study are designated with an asterisk in front of the taxa name. The Roman numerals presented in the taxa names in the phylogenetic tree represent the respective sub/genotype for each isolate, followed by the GenBank identification number, host name, country of isolation, strain designation and year of isolation (if available).

One bird from both the CA02 medium dose and BE08 high dose groups and two birds from the CA18 high dose group had necrotic and necrohemorrhagic areas on the comb and legs (Fig. 3A and C). Two birds from the BE08 high dose group presented neurological signs; one had torticollis and ataxia (Fig. 3B) while the other showed head tremors. A single bird in the CA02 high dose group displayed labored breathing and gasping. Two birds from the CA02 low dose group showed signs of mild lethargy and conjunctivitis at 4 and 5 DPI. All birds from the CA18, CA02, and BE08 high dose groups succumbed to infection or were euthanized by 4, 5, and 5 DPI, with mean death times (MDT) of 4, 4.2, and 5 days, respectively. All birds infected with the medium dose of the study viruses died about a day later for each of the respective groups, except one bird from the BE08 medium dose group, which survived until 9 DPI (MDT 4.6, 5.2, and 6.4 days for CA18, CA02, and BE08, respectively). In the CA02 low dose group, two birds died by 6 DPI, and the remaining three birds, after presenting moderate lethargy, succumbed to infection at 9 DPI (n = 1) and 11 DPI (n = 2) with MDT for the group of 8.4 days.

3.4. Clinical signs in adult hens

Survival curves demonstrating the mortality of 62-week-old (adult) hens after oculo-nasal challenge with the three viruses at three different challenge doses are presented in Fig. 2. All directly challenged adult birds showed clinical signs with the exception of the low dose BE08 group. The onset of clinical signs was observed in the CA18 high dose

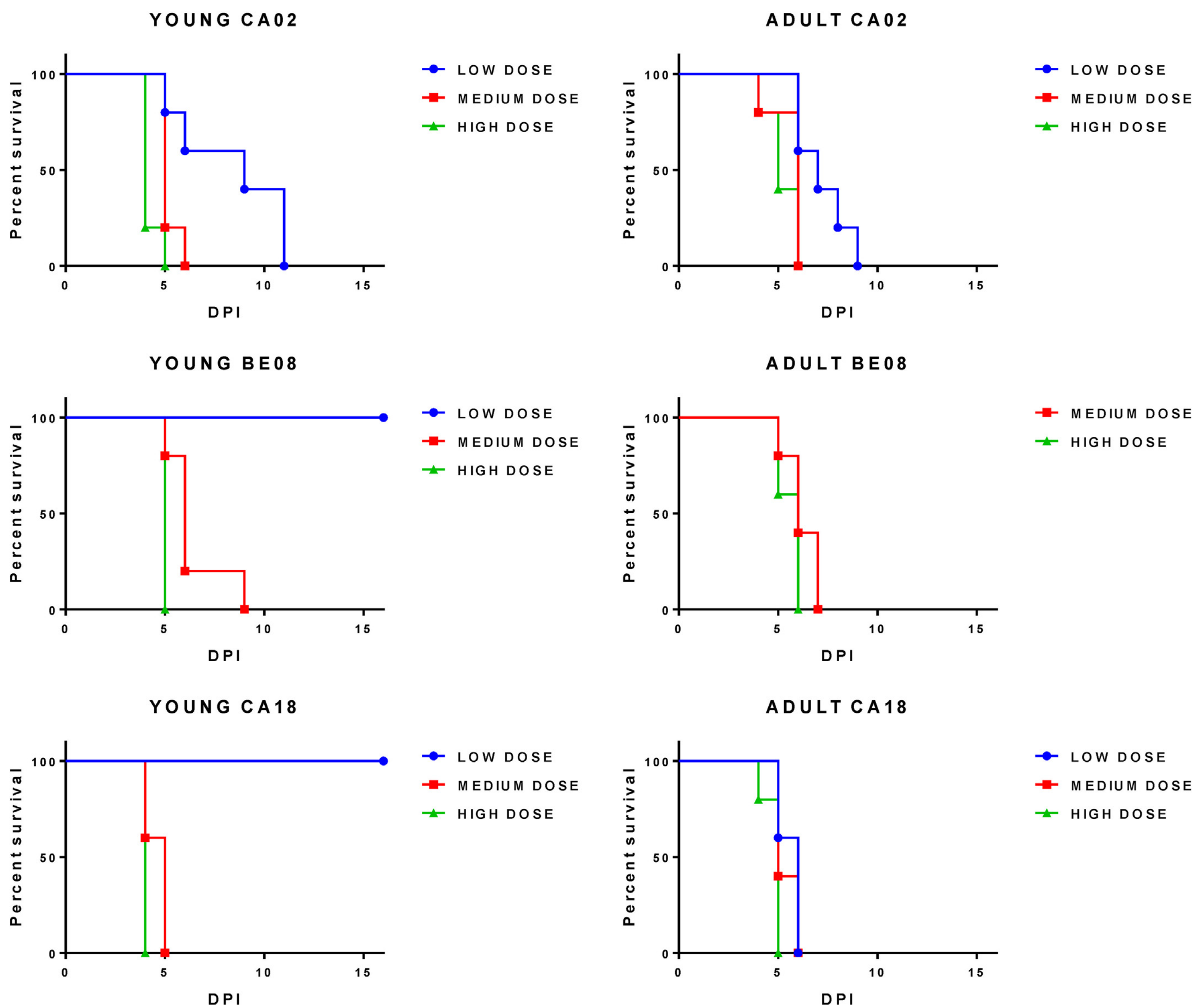


Fig. 2. Survival of three- and 62-week-old chickens experimentally infected with a low, medium, and high dose of challenge NDV (CA02, BE08, and CA18) via intranasal/intraocular inoculation and observed over a 16-day period.

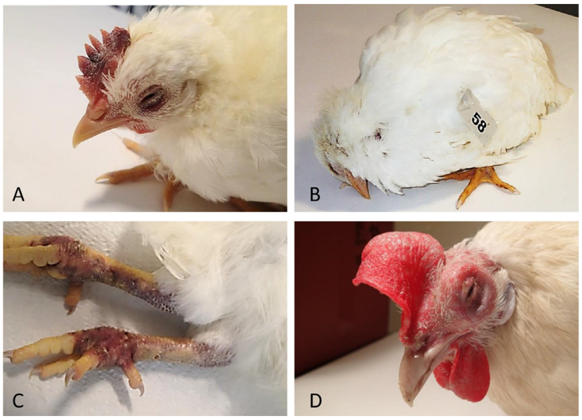


Fig. 3. Clinical signs observed in three- and 62-week-old chickens experimentally infected with virulent NDV (CA02, BE08, and CA18) via intranasal/intraocular inoculation. (A) Severe lethargy and necrotic areas on the comb, 3-week-old chicken. (B) Severe lethargy and torticollis, 3-week-old chicken. (C) Necrohemorrhagic areas on the legs, 3-week-old chicken. (D) Conjunctivitis with periorbital swelling, 62-week-old hen.

group as early as 2 DPI, consisting of moderate lethargy and unilateral conjunctivitis. At 3 and 4 DPI, the birds from the medium dose groups showed mild lethargy and moderate conjunctivitis, whereas moderate lethargy and severe bilateral conjunctivitis was observed in the high dose groups. In addition, all birds with conjunctivitis presented periocular swelling that was more pronounced in the high dose groups (Fig. 3D). Birds challenged with all doses of BE08 and CA18, and CA02 high dose also had diarrhea. The birds in the BE08 low dose group did not present clinical signs through 4 DPI, when a compromised isolator required the experiment for this group to be ended. At 5 and 6 DPI, moderate to severe lethargy with moderate to severe conjunctivitis and periocular swelling was observed in all groups. All birds from the CA18, CA02, and BE08 high dose groups succumbed to the vNDV infection by 5, 6, and 7 DPI, respectively (MDT of 4.8, 5.2, and 5.6 days); all birds from the medium dose groups died by 6, 6, and 7 DPI, respectively (MDT of 5.4, 5.6, and 5.6 days). The birds in the CA18 and CA02 low dose groups died by 6 and 8 DPI, respectively. Three birds from the CA02 low dose group had labored breathing and excessive oronasal discharges at 7 and 8 DPI.

In both young and adult birds, no clinical signs were observed in the control groups. No significant differences in mortality rates were observed between the birds in corresponding dose groups of the viruses within each of the ages. Similarly, no significant differences in mortality were observed between bird ages of the same dose groups with the same virus.

3.5. Gross lesions in three-week-old chickens

Upon examination, the CA18 high dose birds euthanized for tissue collection at 1 DPI lacked lesions. At 2 DPI, all three necropsied CA18 high dose birds had enlarged cecal tonsils and one had splenomegaly. At 3 and 4 DPI, the high dose birds from all three challenge groups had skeletal muscle and organ congestion, suggestive of dehydration. In most birds, a mottled enlarged spleen, swollen kidneys with lobular surface pattern, an enlarged hemorrhagic thymus, a flaccid swollen bursa, hemorrhages in the proventriculus and larynx, eyelid hemorrhages, and necrosis and hemorrhages in the comb were present (see Supplemental Fig. S2). Also, commonly observed in the birds infected with the CA18 virus were hemorrhages in the cecal tonsils and greenish intestinal content. In the birds infected with BE08, in addition to the cecal tonsils, hemorrhages and necrosis were also observed in the ileum. Greenish content and intestinal hemorrhages were present in the birds infected with the CA02 virus. In the contact birds at 5 DPP, the spleens appeared atrophic. No gross lesions were observed in the control birds examined at 2 DPI.

3.6. Gross lesions in adult hens

Birds infected with the high dose of the CA18 virus showed no significant lesions when examined at 1, 2, and 3 DPI (with the exception of one bird at 3 DPI with pinpoint hemorrhages in the proventriculus and one bird with yellow-brownish foamy feces). Upon gross examination at and after 4 DPI of birds infected with all three viruses, general organ congestion, enlarged and mottled spleens, swollen kidneys with a lobular surface pattern, hemorrhages in the proventriculus, green intestinal content, hemorrhages in the larynx and in the upper trachea, comb cyanosis and discoloration, eyelid hemorrhages, airsacculitis, and hemorrhages in yolk follicles (see Supplemental Fig. S2) (some with necrosis) were observed in most necropsied birds. Hemorrhages in the cecal tonsils were present in some of the birds from the BE08 groups, whereas hemorrhages in the intestines were observed in the birds infected with the CA18 virus. The spleens in the contact birds appeared atrophic and pale. No gross lesions were observed in the control group.

3.7. Microscopic lesions and viral antigen staining in tissues

Microscopic lesions and viral antigen staining in tissues of both young and adult chickens inoculated with any of the three NDV when examined at 4–5 DPI were similar, widespread, and consistent with previous descriptions (Ecco et al., 2011; Moura et al., 2016; Pandarangga et al., 2016; Sa e Silva et al., 2013; Susta et al., 2015; Wakamatsu et al., 2006a, 2006b). Detailed description of the microscopic lesions and the lesions observed in chickens inoculated with CA18 from 1 to 3 DPI will be presented elsewhere. Briefly, the most common microscopic lesions were present in the lymphoid organs (spleen, bursa, and thymus) and mucosa-associated lymphoid tissues such as those in the conjunctiva, cecal tonsils, bronchus-associated lymphoid tissues (BALT), and gut-associated lymphoid tissues (GALT), and consisted of mild to severe lymphocellular necrosis and apoptosis. Other common lesions included: necrosis of the epithelium of the nasal turbinates, Harderian glands, trachea, larynx, proventriculus, and intestine; interstitial pneumonia; airsacculitis; submucosal edema, congestion, hemorrhage and mononuclear inflammatory cell infiltration in the connective tissue of the eyelid and comb; areas of gliosis and Purkinje cell necrosis in the cerebellum and neuronal necrosis and gliosis in the cerebrum; focal renal tubular necrosis; and mononuclear cell infiltrates in testis.

NDV-nucleoprotein antigen staining was intracytoplasmic and extracellular in areas of necrosis and was present in many organs and tissues of chickens inoculated with all three examined viruses. Scoring of viral antigen staining and description of stained cell types are found in Table 1. The most extensive and intense viral antigen staining was observed in the mucosa and submucosa of nasal turbinates, sinuses, trachea, larynx, eyelids, comb, proventriculus, lymphoid organs (spleen, thymus, bursa), and mucosa-associated lymphoid tissues (Fig. 4). Immunostaining occurred in epithelial cells, infiltrating mononuclear cells (lymphocytes and macrophages), and vascular endothelial cells. Staining was also present in renal tubular epithelial cells, pancreatic exocrine cells, Kupffer cells in the liver, cardiomyocytes, neurons in the brain, seminiferous epithelium in testis, and periglandular large cells in the esophagus. In many of these organs, positive staining was also detected in infiltrates of lymphocytes and macrophages. In the lung, positive cells, most likely macrophages, were scattered in alveolar septa. Virus-specific antigen staining was absent from all tissues from non-challenged control birds.

Examination of the reproductive tract for NDV-specific staining was carried out on the adult hens euthanized at 4–5 DPI. Infected birds had multifocal areas of necrosis with hemorrhages and mononuclear

Table 1
Distribution of NDV antigen in tissues collected from three- and 62-week-old chickens experimentally infected with a medium and high dose of challenge NDV (CA02, BE08, and CA18) via intranasal/intraocular inoculation. Tissues were collected at 4–5 days post-inoculation. The scoring is expressed in the form of chicken 1/chicken 2.

Tissue	3-week-old			62-week-old			Viral antigen stained cell types
	CA02	BE08	CA18	CA02	BE08	CA18	
Nasal turbinate	++++/+++	++++/++++	++++/++++	++++/+++	++++/++++	NA/++	Epithelial cells and infiltrating mononuclear cells
Trachea	++++/+++	++++/++	++++/+++	-/+	+++/++++	++++/++++	Epithelial cells and infiltrating mononuclear cells, vascular endothelial cells
Larynx	++/+++	++/++++	++++/++++	+++/++	+++/+++	NA/++++	Epithelial cells and infiltrating mononuclear cells
Lung	++/+++	++/+	+++/++++	++/++	++/+++	+++/+++	Lymphoid aggregates present in bronchus-associated lymphoid tissues. Macrophages scattered in alveolar septa
Air sacs	+/+	+++/NA	+++/+++	++/++	NA/++	++/+++	Epithelial cells and infiltrating mononuclear cells
Comb	++++/++++	++++/++++	++++/+++	++/ +++	+++/+++	NA/++	Vascular endothelial cells, mononuclear cells, and necrotic debris
Eye lid	++++/++++	++/++++	++++/++++	++++/++++	++++/++++	+++ /++++	Vascular endothelial cells, interstitium, and epithelial cells
Heart	++/+	-/-	++/+	NA/-	-/-	-/-	Myocardiocytes, epicardium cells, and infiltrating mononuclear cells
Brain	++/++	+/+	++/++	+/+	+/+	+/+	Neurons, Purkinje cells, ependymal cells, glial cells, choroid plexus
Esophagus	+/+	-/-	+++/++	-/+	NA/++	-/-	Periglandular large cells, infiltrating mononuclear cells
Proventriculus	+++/+++	++++/++++	++++/++++	+++/++++	++++/++++	+++/++++	Epithelial cells of the mucosa and gland, mononuclear cells infiltrating mucosa and submucosa
Duodenum	+++/++	++/++	+++/+++	+/+	++/+++	++/+++	Epithelial cells and mononuclear cells infiltrating the mucosa and submucosa.
Cecal tonsils	+++/+++	++++/+++	++++/++++	+++/+++	+++/++++	+++/++++	Mononuclear cells infiltrating mucosa and submucosa
Pancreas	++/++	-/+	++/++	-/++	++/++	-/++	Acinar cells and mononuclear cells
Liver	++/++	+++/+	++/++	++/+	+/+	+/+	Kupffer cells and mononuclear cells
Kidney	+/++	+/-	++/+	+/+	++/++	-/+	Tubular epithelial cells and interstitial mononuclear infiltrate cells
Adrenal gland	+/+	++/-	-/+	NA/NA	++/+	NA/NA	Corticotrophic, corticotropic cells, and infiltrating mononuclear cells
Spleen	++++/++++	++++/+++	+++/++++	++/++	++/++++	-/NA	Lymphocytes and macrophages within areas of necrosis
Thymus	++++/++++	++++/+++	+++/++++	NA/NA	NA/NA	NA/NA	Lymphocytes and macrophages within areas of necrosis
Cloacal bursa	+++/++	+/++	+++/++	NA/NA	NA/NA	NA/NA	Lymphocytes and macrophages within areas of necrosis
Harderian gland	++++/++	++/+	NA/+++	+++/NA	NA/+++	+/+++	Epithelial cells and infiltrating mononuclear cells
Skeletal muscle	-/-	-/NA	-/-	-/-	NA/+	NA/-	Infiltrating mononuclear cells
Feathers	++/++	++/++	++/++	-/+	+/-	-/-	Feather pulp
Testis	+/+	-/NA	++/++	NA/NA	NA/NA	NA/NA	Infiltrating mononuclear cells
Ovaries	NA	NA	NA	+++/+++	++++/NA	+++/+	Tegument/interstitial tissue, granulocytes, endothelial cells, and infiltrating mononuclear cells
Infundibulum	NA	NA	NA	-/++	++/NA	++/+++	Ciliated epithelial cells, tubular glands, and scattered infiltrating mononuclear cells in the lamina propria
Magnum	NA	NA	NA	++/++	+/NA	-/-	Ciliated epithelial cells and infiltrating mononuclear cells
Isthmus	NA	NA	NA	-/+	+/NA	-/++	Ciliated epithelial cells and infiltrating mononuclear cells
Shell gland	NA	NA	NA	+/+	+/NA	++/-	Ciliated epithelial cells and infiltrating mononuclear cells

NA = not available; – = no IHC signal present; + = rare cells positive on IHC; ++ = positive cells seen, < 50% of HPF; +++ = positive signal seen in 50% to 75% of HPF; ++++ = abundant positive signal in > 75% of HPF. HPF, high-power field, ×400 magnification.

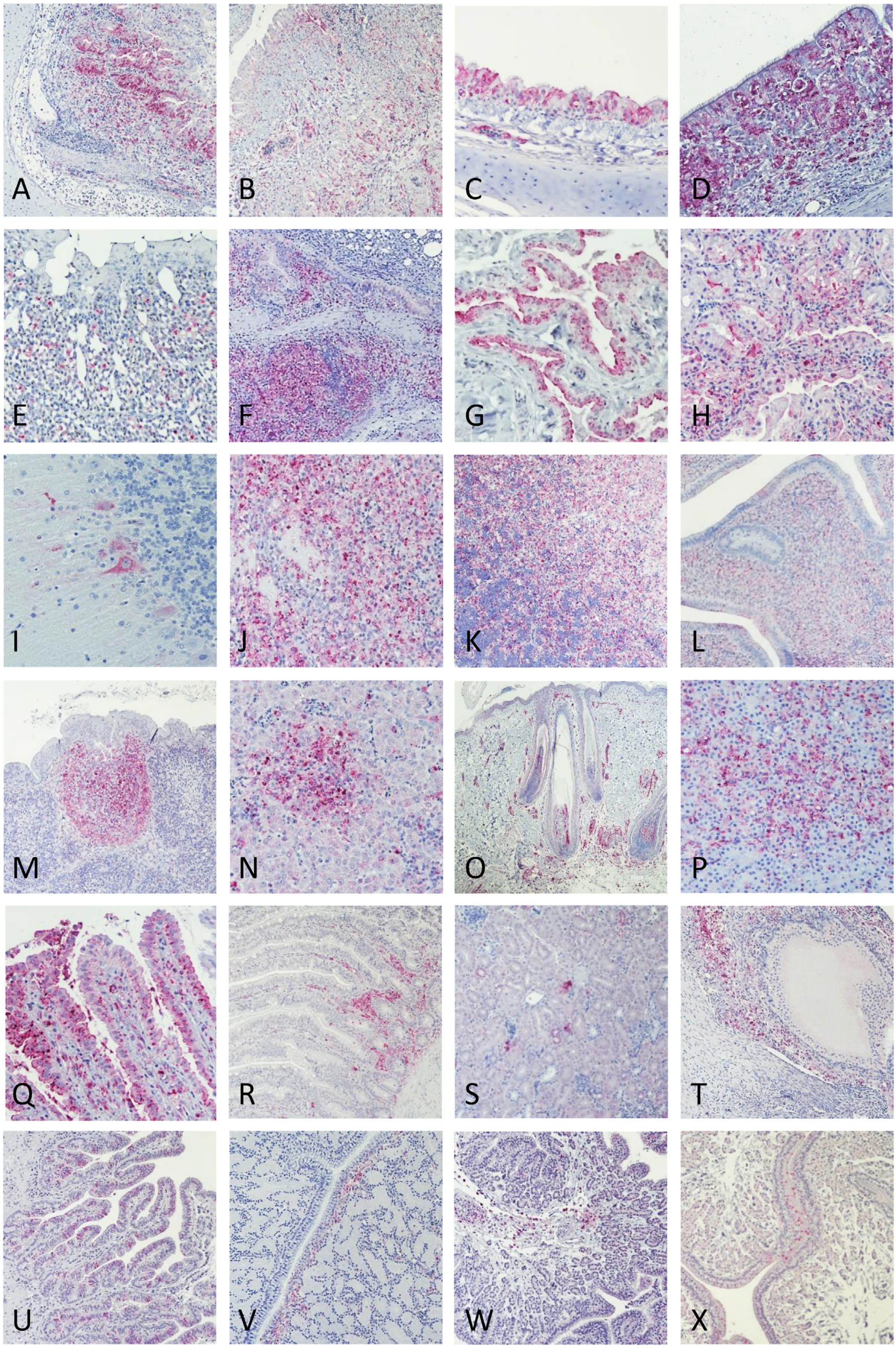


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Fig. 4. Immunohistochemical staining for NDV antigen in tissues collected from three-and 62-week-old chickens experimentally infected with NDV via intranasal/intraocular inoculation. Tissues were collected at 4 days post challenge. Viral antigen is stained in red. (A) Nasal epithelium. Viral antigen staining in epithelial cells, infiltrating mononuclear cells and cellular debris; (B) Eye lid. Viral staining in conjunctival associated lymphoid cells; (C) Trachea. Viral staining in tracheal epithelium and vascular endothelial cells; (D) Larynx. Viral staining in respiratory epithelial cells and infiltrating mononuclear cells; (E) Lung. Viral staining in mononuclear cells in alveolar septa; (F) Bronchus-associated lymphoid tissues (BALT). Viral staining in lymphoid aggregates; (G) Air sac. Viral staining in epithelial cells; (H) Harderian gland. Viral staining in glandular epithelial cells and infiltrating mononuclear cells; (I) Cerebellum. Viral staining in Purkinje cells and glial cells; (J) Spleen. Viral staining in lymphocytes, macrophages and cellular debris; (K) Thymus. Viral staining in lymphocytes and macrophages; (L) Cecal tonsils. Viral staining in lymphocytes and macrophages; (M) Bursa. Viral staining in lymphocytes and macrophages; (N) Liver. Viral staining in Kupfer cells and infiltrating mononuclear cells; (O) Skin. Viral staining in feather follicle epithelium and perifollicular cells, vascular endothelium and infiltrating mononuclear cells; (P) Pancreas. Viral staining in acinar cells and infiltrating mononuclear cells; (Q) Proventriculus. Viral staining in mucosal epithelial cells and infiltrating mononuclear cells; (R) Duodenum. Viral staining in epithelial cells and mononuclear cells infiltrating the lamina propria; (S) Kidney; Viral staining in tubular epithelial cells and infiltrating mononuclear cells; (T) Ovary. Viral staining in thecal layer and infiltrating mononuclear cells in the parenchymal stroma; (U) Infundibulum. Viral staining in ciliated epithelial cells; (V) Magnum. Viral staining in epithelial cells of luminal surface and tubular gland cells; (W) Isthmus. Viral antigen staining in infiltrating mononuclear cells in the parenchymal stroma; (X) Shell gland. Viral antigen in epithelial cells.

inflammatory infiltration in the ovary. Mild edema and mononuclear cells infiltration was present in the infundibulum. In the magnum and isthmus, lesions consisted of focal necrosis of tubular glands epithelial cells with inflammatory cells infiltration. In the ovary, viral antigen staining was detected in granulosa cells within atretic follicles, luteinized (foamy) cells and mesothelial cells. In the infundibulum, immunostaining was detected in ciliated epithelial cells, tubular glands, and scattered monocytes in the lamina propria. In the magnum, isthmus, and shell gland, viral antigen staining was focally detected in the epithelial ciliated cells (Fig. 4).

3.8. Virus shedding and serology in three-week-old chickens

At 1 DPI, two, four, and five birds in BE08, CA02, and CA18 medium dose groups, respectively, were shedding virus through the oral route. One bird from the CA18 medium dose group and three birds from the CA18 high dose group shed virus by the cloacal route at low titers (Table 2, Fig. 5). At 1 DPI, all birds in the high dose groups shed virus by the oral route with titers ranging from $10^{3.8}$ and $10^{4.7}$EID$_{50}$/ml. At 2 DPI (high dose) and at 3 and 4 DPI (medium dose), the birds inoculated with CA18 shed significantly higher titers ($P = 0.002$, $P = 0.019$, $P = 0.046$, respectively) by the oral route compared to the same dose group inoculated with BE08, but not to the group inoculated with CA02. Based on the backtiter results of the inocula, the BE08 birds received the lowest challenge dose which probably affected the observed results (Table S1). The shed virus titers increased during the following DPI in all groups, peaking at 4 DPI (Fig. 5). The virus titers at 4 DPI were as high as $10^{8.1}$ and $10^{8.0}$ EID$_{50}$/ml in OP and CL swabs, respectively, in all groups infected with the medium and high doses (Table 2). The low dose CA02 challenged group had 2 out of 5 birds with detectable virus at 2 and 3 DPI by the oral route and 3 out of 5 birds shed virus at 4 DPI by the oral route. Eventually all the birds in these groups became infected. Shedding by both oral and cloacal routes was observed in the CA18 and BE08 low dose groups at a low level ranging from $10^{2.1}$ to $10^{2.9}$ EID$_{50}$/ml only at 7 DPI, but not at 1–4 DPI, and these birds were all serologically negative as demonstrated by the HI test performed at 16 DPI (14 DPP) (Table 2). The chickens from these two groups were the only birds that survived the experiment. The rRT-PCR results at 7 DPI appear to be an anomaly as all other results suggest the birds were not infected, and cross contamination of the 7 DPI samples cannot be discounted.

3.9. Virus shedding in adult hens

Virus shedding was detected as early as 1 DPI by the oral route from all birds in medium and high dose groups, except 3 birds in the BE08 medium dose group, with mean titers ranging between $10^{3.1}$ and $10^{5.8}$ EID$_{50}$/ml, respectively. Similar to young birds, viral shedding at 1 DPI by the cloacal route was detected in low titers from only a few birds (4 of 37 birds) in the medium and high dose groups (Table 2, Fig. 6). The amounts of virus shed by both routes gradually increased during the following days in all groups. The peak of shedding from the adult hens in high dose groups was also at 4 DPI (Fig. 6) with shedding titers of $10^{6.0–7.8}$ EID$_{50}$/ml and $10^{5.2–6.9}$ EID$_{50}$/ml through the OP and CL routes, respectively. At 4 DPI, the birds infected with the high dose CA18 shed significantly more virus through the oral route compared to the birds infected with CA02 ($P = 0.005$), but no significant difference was found when compared to the BE08 group. The CA02 and the CA18 low dose groups had 2 out of 5 and 4 out of 5 birds, respectively, positive at 1 DPI, and all the birds were positive by 4 DPI with shedding titers of $10^{5.2–7.1}$ EID$_{50}$/ml from the OP route and $10^{3.3–6.8}$ EID$_{50}$/ml from the CL route. The BE08 low dose group had no positive samples at 4 DPI when the experiment for this group was prematurely ended.

3.10. Comparison of viral shedding between three- and 62-week-old chickens

Significant difference in viral shedding through the cloacal route between the young and adult birds was determined only in the groups inoculated with the CA18 high dose at 2 and 3 DPI ($P = 0.043$ and $P = 0.035$, respectively). The young birds shed significantly more virus ($10^{5.1–7.3}$ EID$_{50}$/ml) than the adult hens ($10^{3.5–5.8}$ EID$_{50}$/ml).

3.11. Transmission of the studied NDV in three-week-old chickens

To assess virus transmission, three, SPF, 3-week-old chickens were added to each experimental group at 2 DPI. All contact birds (except in BE08 and CA18 low dose groups) showed clinical signs. The first clinical signs were observed at 3 DPP in the CA02 (n = 2) and CA18 (n = 3) high dose groups and consisted of mild lethargy. One bird in the CA02 group also displayed labored breathing and head tremors. At 4 and 5 DPP, all birds from the affected groups presented mild (BE08 medium dose group), mild to moderate (CA02 and CA18 medium dose groups), and moderate to severe lethargy (all high dose groups). All affected birds also showed mild to moderate bilateral conjunctivitis. All contact birds that showed clinical signs succumbed to vNDV infection: the CA18, CA02, and BE08 high dose groups by 5, 6, and 7 DPP, respectively, and the medium dose groups a day later for each of the virus groups. The titers of the virus shed through the OP route from the birds in the medium and high dose groups ranged from $10^{3.1}$ to $10^{5.1}$ EID$_{50}$/ml at 2 DPP with the highest titers detected in the CA18 group. Virus shedding detected through CL swabs was slightly lower, mainly in CA02 medium dose group ($10^{1.9}$ EID$_{50}$/ml); whereas, in the other two groups, the shedding varied from $10^{2.7}$ to $10^{3.3}$ EID$_{50}$/ml at 2 DPP. At 5 DPP, birds infected with the medium dose of CA18 shed up to $10^{8.0}$ and $10^{7.6}$ EID$_{50}$/ml detected in both OP and CL swabs, respectively. On the same day, the only surviving bird in the high dose CA18 group shed virus at $10^{7.9}$ and $10^{8.1}$EID$_{50}$/ml detected in OP and CL swabs, respectively (Fig. 5, Table 2).

Only the birds in the CA02 low dose group showed clinical signs with mild to moderate lethargy at 7 DPP and died during the following two days. Similar to the directly challenged birds from the CA18 and

Table 2
Susceptibility and transmission of virulent NDV (CA02, BE08, and CA18) and mean viral shedding in three- and 62-week-old chickens experimentally infected with a low, medium, and high dose of challenge viruses via intranasal/intraocular inoculation.

Experiment and virus dose	No. of virus-positive birds/total number of birds and mean shedding titers[a,b]										Serology
	1 DPI		2 DPI		3 DPI		4 DPI/ 2 DPP		7 DPI/ 5DPP		16 DPI/14 DPP
	OP	CL	OP	CL	OP	CL	OP	CL	OP	CL	
three-week-old											
CA02 low dose	0/5	0/5	2/5 (1.8)	0/5	2/5 (2.9)	2/5 (3.5)	3/5 (4.9)	2/5 (6.0)	3/3 (4.8)	2/3 (2.6)	NA
CA02 low dose contacts							2/3 (2.6)	0/3	3/3 (3.8)	3/3 (2.9)	NA
CA02 medium dose	4/5 (2.1)	0/5	5/5 (3.8)	3/5 (3.0)	5/5 (5.2)	5/5 (4.6)	5/5 (6.7)	5/5 (6.5)	NA	NA	NA
CA02 medium dose contacts							3/3 (3.1)	2/3 (1.9)	3/3 (5.9)	3/3 (5.1)	NA
CA02 high dose	5/5 (4.0)	0/5	5/5 (5.2)	5/5 (4.4)	5/5 (6.5)	5/5 (6.5)	1/1 (7.6)	1/1 (7.3)	NA	NA	NA
CA02 high dose contacts							3/3 (3.6)	3/3 (2.6)	3/3 (6.9)	3/3 (6.3)	NA
BE08 low dose	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	4/5 (2.1)	2/5 (2.3)	0/5
BE08 low dose contacts							0/3	0/3	2/3 (2.6)	2/3 (1.9)	0/3
BE08 medium dose	2/5 (3.0)	0/5	4/5 (3.9)	2/5 (3.1)	5/5 (4.4)	5/5 (4.2)	5/5 (5.8)	5/5 (5.6)	1/1 (7.0)	1/1 (4.8)	NA
BE08 medium dose contacts							3/3 (3.4)	2/3 (2.7)	3/3 (6.4)	3/3 (5.5)	NA
BE08 high dose	5/5 (3.8)	0/5	5/5 (4.7)	5/5 (4.2)	5/5 (6.1)	5/5 (5.8)	5/5 (6.9)	5/5 (7.3)	NA	NA	NA
BE08 high dose contacts							3/3 (4.3)	2/3 (3.0)	3/3 (7.1)	3/3 (6.9)	NA
CA18 low dose	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	5/5 (2.6)	2/5 (2.5)	0/5
CA18 low dose contacts							0/3	0/3	3/3 (2.6)	2/3 (2.2)	0/3
CA18 medium dose	5/5 (3.7)	1/5 (1.9)	5/5 (4.7)	4/5 (4.1)	5/5 (6.7)	5/5 (6.4)	4/4 (8.1)	4/4 (7.8)	NA	NA	NA
CA18 medium dose contacts							3/3 (5.1)	2/3 (3.3)	3/3 (8.0)	3/3 (7.6)	NA
CA18 high dose[c]	14/14 (4.7)	3/14 (2.6)	11/11 (6.0)	11/11 (5.1)	8/8 (7.5)	8/8 (7.3)	2/2 (7.5)	2/2 (8.0)	NA	NA	NA
CA18 high dose contacts							3/3 (4.3)	3/3 (3.3)	1/1 (7.9)	1/1 (8.1)	NA
62-week-old											
CA02 low dose	2/5 (3.0)	0/5	2/5 (5.2)	0/5	3/5 (4.5)	2/5 (2.9)	5/5 (5.2)	3/5 (3.3)	3/3 (6.1)	3/3 (5.8)	NA
CA02 low dose contacts							2/2 (2.9)	0/2	2/2 (6.8)	2/2 (6.1)	NA
CA02 medium dose	5/5 (4.0)	1/5 (1.9)	5/5 (4.8)	2/5 (2.3)	5/5 (5.1)	5/5 (4.0)	5/5 (6.6)	5/5 (5.2)	NA	NA	NA
CA02 medium dose contacts							1/2 (2.7)	0/2	2/2 (6.0)	2/2 (5.4)	NA
CA02 high dose	5/5 (5.3)	0/5	5/5 (5.7)	2/5 (4.8)	5/5 (5.9)	5/5 (4.8)	4/4 (6.0)	4/4 (5.2)	NA	NA	NA
CA02 high dose contacts							2/2 (3.8)	1/2 (2.6)	2/2 (6.9)	2/2 (6.6)	NA
BE08 low dose	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	ND[d]	ND[d]	NA
BE08 low dose contacts							0/2	0/2	ND[d]	ND[d]	NA
BE08 medium dose	2/5 (3.1)	0/5	5/5 (4.2)	0/5	5/5 (4.9)	3/5 (3.6)	5/5 (6.1)	5/5 (5.2)	NA	NA	NA
BE08 medium dose contacts							2/2 (2.2)	0/2	2/2 (7.0)	2/2 (5.1)	NA
BE08 high dose	5/5 (4.9)	1/5 (1.9)	5/5 (5.2)	4/5 (2.4)	5/5 (5.9)	5/5 (5.0)	5/5 (6.8)	5/5 (6.3)	NA	NA	NA
BE08 high dose contacts							2/2 (3.7)	0/2	2/2 (7.3)	2/2 (6.1)	NA
CA18 low dose	4/5 (2.9)	0/5	5/5 (5.5)	0/5	5/5 (5.9)	5/5 (5.0)	5/5 (7.1)	5/5 (6.8)	NA	NA	NA
CA18 low dose contacts							2/2 (4.0)	1/2 (2.9)	2/2 (7.4)	2/2 (6.1)	NA
CA18 medium dose	5/5 (4.1)	0/5	5/5 (5.4)	2/5 (2.6)	5/5 (6.4)	4/5 (5.4)	5/5 (7.5)	5/5 (6.1)	NA	NA	NA
CA18 medium dose contacts							2/2 (5.2)	1/2 (2.3)	2/2 (7.2)	2/2 (6.9)	NA
CA18 high dose[c]	12/12 (5.8)	2/12 (2.8)	10/10 (5.7)	8/10 (3.5)	8/8 (6.3)	8/8 (5.8)	4/4 (7.8)	4/4 (6.3)	NA	NA	NA
CA18 high dose							2/2 (5.4)	1/2 (2.6)	1/1 (8.3)	1/1 (7.7)	NA

DPI = days post inoculation.
DPP = days post placement.
NA = not available, all birds succumbed to infection before the day of sampling.
[a] Results of testing for NDV in oropharyngeal (OP) and cloacal (CL) swab samples collected from inoculated birds and birds exposed through direct contact. Values in parenthesis are the mean virus titers as determined by rRT-PCR for positive samples and are reported as $\log_{10}$ EID_{50}/ml. The estimated lower limit of detection of the rRT-PCR test was between $10^{1.5}$ and $10^{1.7}$ EID_{50}/ml. The results are presented as the average viral shedding for each day, and samples negative by rRT-PCR were not used in the calculations.
[b] Data represent the number of positive samples/total number of birds tested. Titers of 8 or below were considered negative.
[c] The numbers of the birds in these groups also include the birds from the pathogenesis groups that were sacrificed at 1, 2, and 3 DPI for tissues collection.
[d] Not done, the experiment in this group was ended at 4DPI due to compromised isolator.

BE08 low dose groups, the transmission birds in these groups did not show any clinical signs. They had sporadic positive low-titer rRT-PCR results at 5 DPP (corresponding to 7 DPI) but were also serologically negative at the end of the experiment (14 DPP) (Table 2).

3.12. Transmission of the studied NDV in adult hens

All adult contact birds showed clinical signs, and the first signs appeared at 5 DPP (except the BE08 low dose group birds for which the experiment was ended at 2 DPP). The clinical signs resembled the ones observed in the directly challenged birds, which consisted of lethargy, conjunctivitis, periorbital swelling, and diarrhea that increased in severity by 7 and 8 DPP when all contact birds succumbed to infection. The birds from the CA02 groups presented labored breathing at 8 DPP. The contact birds from the CA18 low dose group had dysphonia. The mean death times for the contact birds were estimated to be 5.5, 6.0, and 6.5 for the CA18, CA02, and BE08 high dose groups, respectively; 7, 7, and 6.5 days for the medium dose groups, respectively. The mean death time for the low dose CA18 and CA02 groups was 6.5 days for both, with no result for the BE08 low dose group. All adult contact birds in the CA02 and CA18 groups shed virus by the OP route at 2 and 5 DPP and by the CL route at 5 DPP (Fig. 6). Birds from the medium and high dose groups shed titers ranging from $10^{2.2}$ to $10^{5.4}$ EID_{50}/ml at 2 DPP detected in OP swabs. At 2 DPP, four out of 12 contact birds in medium and high dose groups shed virus through the cloacal route in low titers, ranging from $10^{2.3}$ to $10^{2.9}$ EID_{50}/ml. At 5 DPP, the contact birds from the groups infected with the medium doses shed virus up to $10^{7.2}$ and $10^{6.9}$ EID_{50}/ml through the oral and cloacal route, respectively. The only remaining living bird in the CA18 high dose group at 5 DPP shed virus at $10^{8.3}$ and $10^{7.7}$ EID_{50}/ml detected in the OP and CL swabs,

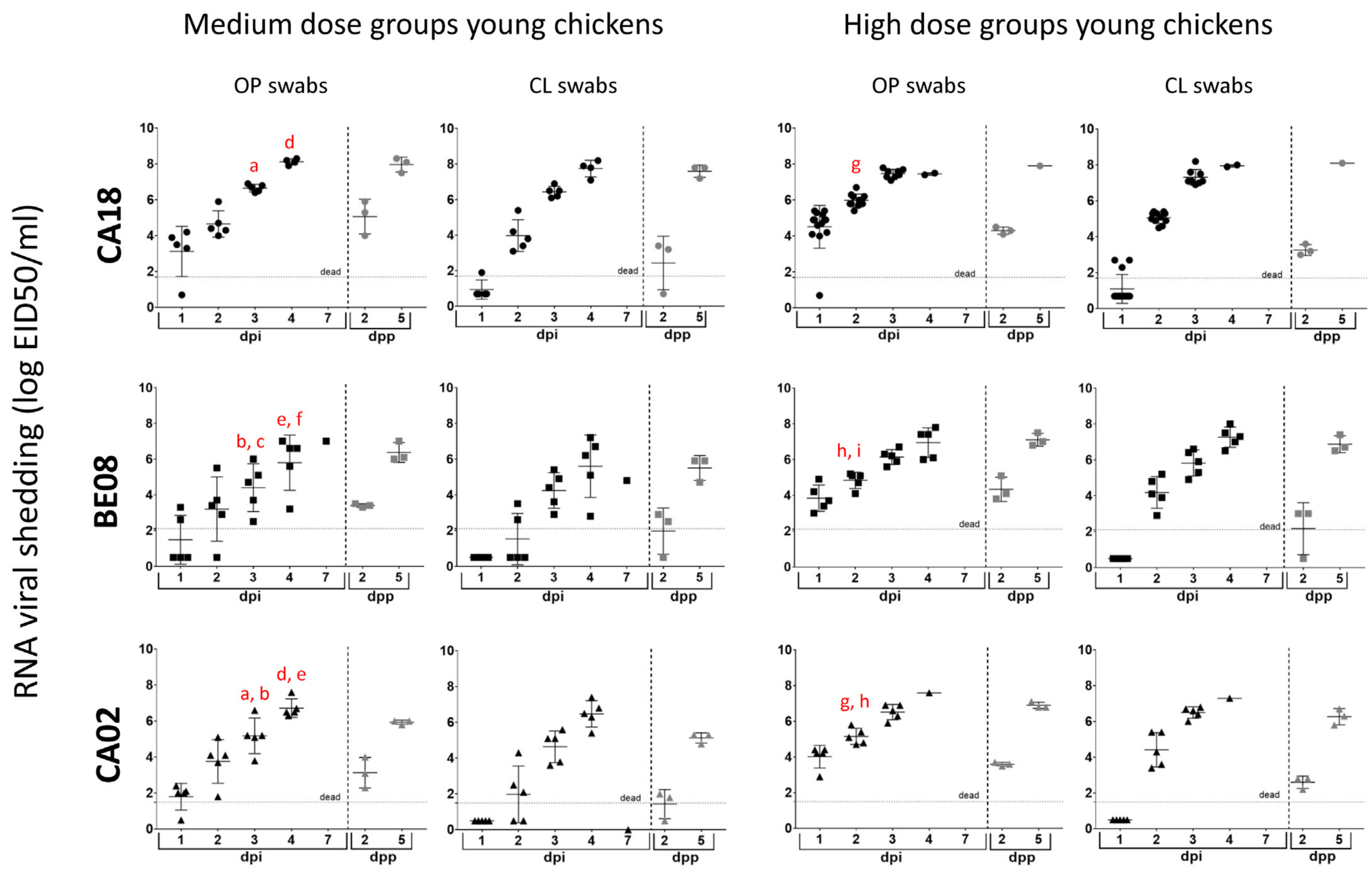

Fig. 5. Mean viral shedding in three-week-old chickens experimentally infected with a medium and high dose of challenge NDV (CA02, BE08, and CA18) via intranasal/intraocular inoculation. Each data point represents NDV titers detected in oropharyngeal (OP) and cloacal (CL) swabs at different days post infection (DPI) or days post placement for contact birds (DPP). Bars represent standard deviations of the mean. All swabs from which virus was not detected were given a numeric value of 1 log below the limit of detection for each of the respective viruses. The limit of detection of rRT-PCR for each virus is presented as a dotted line. Statistically significant differences between the different viral groups are presented with lowercase letters in red font. Differences are presented within the same dose and the same sampling day (a,b,c for medium dose at 3 DPI; d,e,f for medium dose at 4 DPI; g,h,i for high dose at 2 DPI).

respectively. The shedding titers from the high dose CA02 and BE08 groups by both routes at 5 DPP were about a log lower compared to the CA18 group (Fig. 6, Table 2).

3.13. Detection of the studied NDV in tissue samples

High mean virus titers were detected in spleens ($10^{7.3}$ to $10^{8.9}$ EID_{50}/ml), lungs ($10^{6.7}$ to $10^{8.0}$ EID_{50}/ml), and brains ($10^{5.3}$ to $10^{7.15}$ EID_{50}/ml) collected at 4 and 5 DPI from young and adult birds infected with the high or medium dose of the three viruses, as demonstrated by the detection of viral nucleic acid by rRT-PCR (Table 3). In addition, high viral titers were detected in samples collected from the reproductive tract of the sampled adult birds ($10^{5.96}$ to $10^{6.6}$ EID_{50}/ml). No marked variation was observed between the virus titers in the same tissues from the different virus groups within each age (differences less than a log), except in the lungs and the brains of the young birds from the BE08 group, where decreased titers up to $10^{1.5}$ EID_{50}/ml were observed. Although the virus titers in the tissues of the young birds were higher compared to those of the adult birds, in most samples, the variation was less than a log, with the exception of the brain and the spleen samples in CA02 and CA18 groups where in young birds the titers were 1.45–1.65 logs higher than the adult birds (Table 3).

4. Discussion

In this study, we evaluated and described the susceptibility of both young and adult chickens to infection with a low, medium and high dose of the virus causing the 2018–2019 California ND outbreak, the pathogenesis and shedding dynamics of the virus, and its transmissibility to naïve contact chickens. Side-by-side experiments utilizing the genetically close vNDV causing outbreaks in California in 2002–2003 and Belize in 2008 were conducted, and the characteristics of the three viruses were compared. In addition, full fusion gene and complete genome phylogenetic analyses were performed to establish the evolutionary relationships between the studied viruses and other class II NDV. This is the first characterization of the sub-genotype Vb vNDV causing the 2018–2019 California outbreak and the first detailed side-by-side pathogenesis characterization of vNDV in young and adult birds.

The clinical signs presented in birds infected by all three viruses were similar and included mainly lethargy and bilateral conjunctivitis that progressed until all birds succumbed to the virulent infection. Individual birds showed labored breathing, neurological signs, or oronasal discharge. The clinical signs observed in all studied groups also resembled those described in previous characterizations of the CA02 and BE08 viruses (Kapczynski and King, 2005; Susta et al., 2014; Wakamatsu et al., 2006a). A marked difference between the young and adult birds in this study was the presence of severe periocular swelling in the latter, regardless of the challenge virus. A possible explanation for this difference, which was present in the adult birds inoculated with all the viruses, could be the more mature immune system of the adult birds which may be related to the more severe reaction to the infection and the delayed mean death time (Miller and Koch, 2013).

We showed that both young and adult chickens are highly

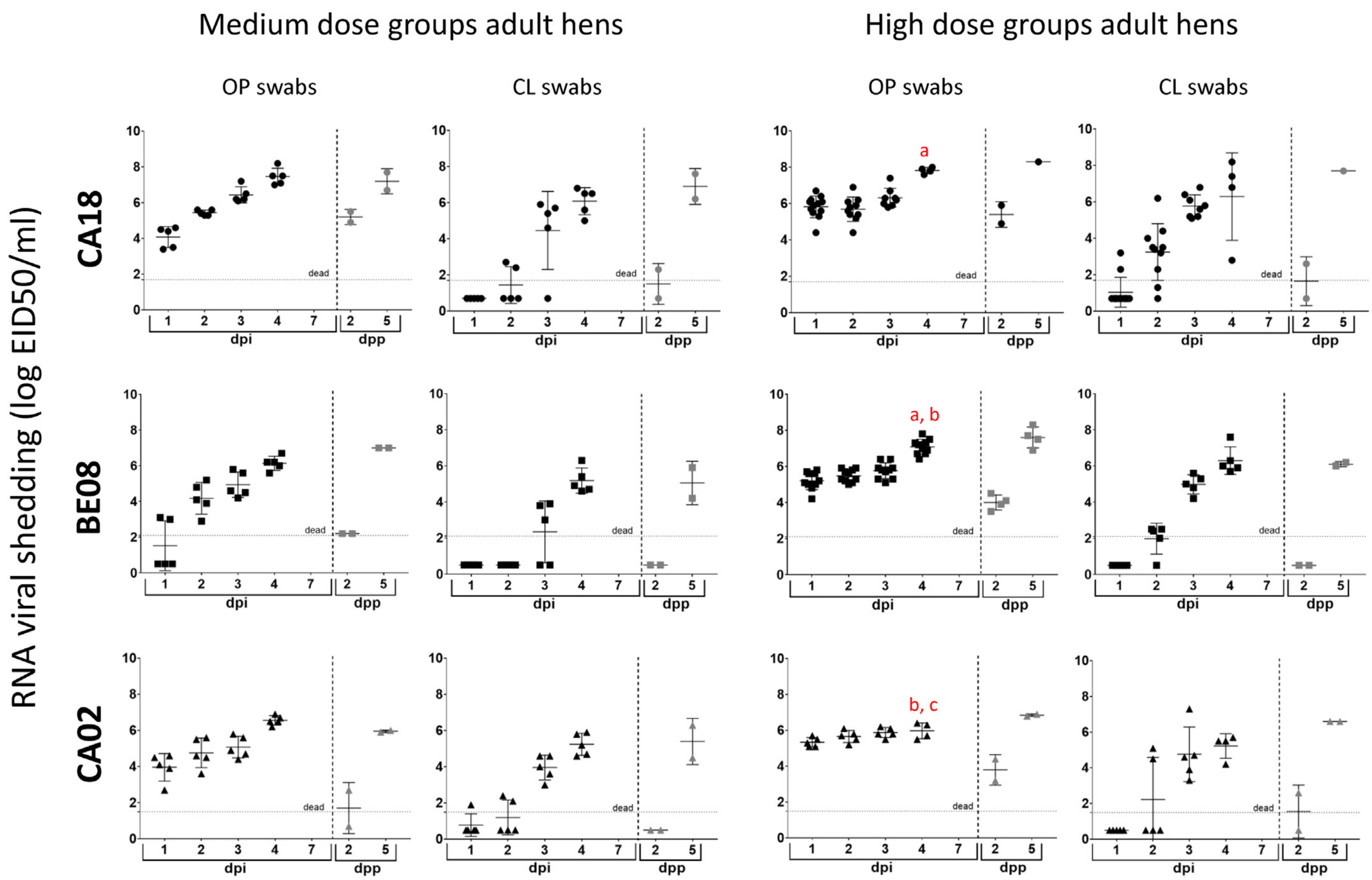

Fig. 6. Mean viral shedding in 62-week-old chickens experimentally infected with a medium and high dose of challenge NDV (CA02, BE08, and CA18) via intranasal/intraocular inoculation. Each data point represents NDV titers detected in oropharyngeal (OP) and cloacal (CL) swabs at different days post infection (DPI) or days post placement for contact birds (DPP). Bars represent standard deviations of the mean. All swabs from which virus was not detected were given a numeric value of 1 log below the limit of detection for each of the respective viruses. The limit of detection of rRT-PCR for each virus is presented as a dotted line. Statistically significant differences between the different viral groups are presented with lowercase letters in red font. Differences are presented within the same dose and the same sampling day (a,b,c for high dose at 4 DPI).

susceptible to infection with all of the three studied viruses. The high titers shed from the birds infected with all three viruses demonstrate that these vNDV are well adapted to poultry and readily infect young and adult chickens in all doses (except the low dose CA18 and BE08 young chickens). Both ages receiving the high dose became infected after inoculation and shed virus as early as 1 DPI, with shedding titers reaching up to 10^8 EID_{50}/ml by 4 DPI. Virus shedding for both ages receiving the medium dose was slightly delayed by one day compared to the high dose group, with peak titers likely to occur at 5 DPI, if swabbing was conducted on that day. For both ages receiving the low dose of CA02, a delay in viral shedding was evident with only 2–3 birds infected by 3 DPI; however, the amount of virus shed was sufficient to transmit to all birds in the cage. The BE08 and CA18 low dose groups were considered to be uninfected based on the negative serology at the end of the experiment.

The transmissibility of the viruses was further confirmed by the demonstrated infection, morbidity, and mortality in the naïve contact birds placed in each dose group for both ages at 2 DPI. In all groups for

Table 3
NDV mean titers (and standard deviation) in lungs, spleen, brain, and reproductive tract samples from three- and 62-week-old chickens experimentally infected with a high or medium dose of challenge NDV (CA02, BE08, and CA18) via intranasal/intraocular inoculation. Tissues were collected at 4 and 5 DPI[a].

Experiment and virus	Virus titer log_{10} EID_{50}/ml			
	Spleen	Lung	Brain	Reproductive tract
three-week-old				
CA02[b]	8.90 (± 0.28)	8.00 (± 0.56)	7.15 (± 0.07)	NA
BE08	8.35 (± 0.21)	6.70 (± 0.00)	5.90 (± 0.14)	NA
CA18[b]	8.75 (± 0.21)	7.95 (± 0.21)	6.95 (± 0.50)	NA
62-week-old				
CA02	8.00 (± 0.57)	7.35 (± 0.07)	5.90 (± 0.42)	6.60 (± 0.57)
BE08	7.80 (± 0.00)	6.90 (± 0.28)	5.45 (± 0.07)	5.96 (± 0.36)
CA18	7.30 (± 0.28)	7.70 (± 0.14)	5.30 (± 0.57)	6.60 (± 0.57)

NA = not available.
[a] Tissues were collected from two birds per group at 4 DPI (with the exception of BE08 adult group where samples were collected at 5 DPI).
[b] One of the three-week-old chickens in each CA02 and CA18 groups from which tissues were collected was infected with a medium dose of challenge virus.

which infection after direct challenge was established (all dose groups except CA18 and BE08 young birds), the viruses readily transmitted to the contact birds even where birds were infected with the low dose of the challenge virus. Similar to the directly infected birds, the amounts of virus shed by the contact birds increased in all groups from 2 to 5 DPP. The efficient transmission to contact birds, even in some of the low dose groups, and the high viral titers shed by both directly inoculated and contact birds, confirms the high adaptation of the viruses to chickens, which would favor their easy dissemination and spread in populations of this species.

All three studied viruses replicated to high titers in the spleens, lungs, and brains of inoculated young and adult chickens and also in the reproductive tract of the adult hens, confirming the systemic nature of the caused infection. Similarly, widespread microscopic lesions and viral antigen staining were observed in tissues from chickens infected with all three viruses. These lesions and sites of virus replication are similar to previously reported in studies with CA02 and BE08 (Susta et al., 2014; Wakamatsu et al., 2006a) and other vNDV (Ecco et al., 2011; Moura et al., 2016; Pandarangga et al., 2016; Susta et al., 2015; Wakamatsu et al., 2006b). The distribution of immunostaining in the reproductive tract of laying hens infected with CA02 was evaluated in a previous study and, similarly to our results, bird to bird variation was also observed (Sa E Silva et al., 2016).

The gross lesions described in the necropsied birds in the present study were suggestive of systemic infection and consistent with those described after infection of SPF birds with the CA02 and BE08 viruses, as well as other virulent class II NDV (Cattoli et al., 2011; Miller and Koch, 2013; Susta et al., 2014; Wakamatsu et al., 2006a). Carvallo et al. report the identification of additional gross lesions in birds submitted for necropsy from field cases during the California 2018–2019 outbreak, including fibrinonecrotizing stomatitis, pharyngitis, esophagitis, and laryngotracheitis (Carvallo et al., 2018). However, with these natural infections, other factors could have affected disease presentation, including exacerbation by other pathogens and adverse environmental conditions (e.g. poor ventilation and high ammonia levels). Reports for avian influenza infections, for example, suggest that factors other than virus strain, such as environmental stress and presence of other infectious agents, may affect the type and severity of the lesions (Dimitrov et al., 2016e; Gharaibeh, 2008; Pantin-Jackwood et al., 2012).

The virus causing the 2018–2019 ND outbreak in California is genetically close to the viruses causing outbreaks in California during 2002–2003, Honduras in 2007, and Belize 2008; however, the phylogenetic analysis suggests that there is no direct causal link between them. Of the available sequences of viruses from sub-genotype Vb, the CA18 virus has highest nucleotide identity to chicken/Honduras/498109–15/2007 (98.2%), followed by chicken/Belize/4224–3/2008 and chicken/USA/CA/212676/2002 (97.9% and 97.4%, respectively). Czeglédi et al. and Herczeg et al. (Czeglédi et al., 2002; Herczeg et al., 2001) reported that the nucleotide sequence change of NDV through natural evolution is estimated to be approximately 1% per decade. The nucleotide distances between the CA18 and these viruses from Central America suggest 18 and 21–26 years of independent evolution, respectively. Considering that the periods between the isolations of the virus causing the current outbreak in California and the viruses isolated in North and Central America during 2002–2008 are shorter, it is not likely that they evolved from each other (Dimitrov et al., 2016c). It is likely that they represent separate introduction events of vNDV that evolved elsewhere (Dimitrov et al., 2016b). The CA18 virus is even more distant from older viruses (1980s and 1990s) that have caused outbreaks in chickens and parrots in the U.S., Mexico, and Central America with nucleotide distances ranging from 4.2% to 7.8%. The CA18 is distinct from sub-genotype Vc NDV known to circulate in Mexico. The high genetic distance and the phylogenetic analysis exclude any direct epidemiological link between these viruses and CA18. With more than 10% nucleotide distance, the CA18 virus is also not related to the virulent viruses of sub-genotype Va maintained in some cormorant colonies in North America. It is doubtful that wild birds played a role in the introduction of the CA18 as viruses from sub-genotype Vb have not been isolated from wild birds in the last 20 years. Based upon the available data, the CA02 virus appears to have been eradicated from California in 2003, and it is highly unlikely that the virus remained undetected and evolved locally in an unknown reservoir, as not a single similar virus has been identified through ongoing surveillance efforts in the United States instituted over the last 15 years. Unfortunately, there are only a few sequences of sub-genotype Vb available between 2003 and 2018. Although multiple cases of ND were reported to the OIE from countries in North and Central America like Honduras, Nicaragua, Costa Rica, and Mexico, between 2005 and 2017 (OIE, 2018), there is no genetic data available from most of these outbreaks. Historic and prospective virus and surveillance data from the Americas may allow a more detailed molecular epidemiological inference of the origin of the CA18 virus.

Our work shows that there are no major differences in the clinical disease and transmissibility between the three studied viruses. The identified similarities in susceptibility of chickens, shedding titers, clinical signs, mortality, transmissibility, and genetic characteristics among the studied viruses allow extrapolation of the existing literature for CA02 to facilitate the control of the current ND outbreak in southern California. Similar to the California 2002–2003 ND outbreak, wild birds likely play, at most, a minor role in the epidemiological process of the disease during the current outbreak, as vNDV has not yet been identified in wild birds. During the California 2002–2003 ND outbreak, vNDV was identified in non-poultry species by rRT-PCR in only 57 out of almost 82,000 samples (Crossley et al., 2005; Kinde et al., 2005). In contrast to the 1971–1973 ND when psittacines have been suggested to play a major role in the outbreak, the incidence in psittacines and in pigeons during the 2002–2003 outbreak, was as low as 0.12% and 0.21%, respectively, of all samples collected from these species (Kinde et al., 2005). In addition, all positive samples from non-poultry species during the 2002–2003 California outbreak were collected on premises either on or within a 1 km radius of known infected chicken premises. During that outbreak, the virus caused clinical disease and mortality in commercial chickens and turkeys (Kapczynski and King, 2005; Wakamatsu et al., 2006a). The CA18 virus has been primarily detected in exhibition backyard poultry but recently spread to four commercial flocks, and disease control and surveillance efforts continue. The potential of further spread of vNDV remains an ongoing threat, and because vaccine strategies have changed in the last 15 years, an updated evaluation of the vaccine protection for commercial poultry species from challenge with the CA18 is warranted.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2019.03.010.

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